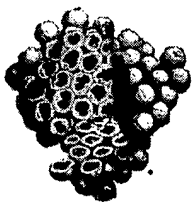

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Lentiviral Vector Transduction of Hematopoietic Stem Cells that Mediate Long-Term Reconstitution of Lethally Irradiated Mice

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Lentiviral Vector Transduction of Hematopoietic Stem Cells that Mediate Long-Term Reconstitution of Lethally Irradiated Mice

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Key Words. *Lentiviral vector · VSV-G · Sca-1⁺c-Kit⁺Lin⁻ cells · Transplantation*

ABSTRACT

Lentiviral vectors efficiently transduce human CD34⁺ cells that mediate long-term engraftment of nonobese diabetic/severe combined immunodeficient mice. However, hematopoiesis in these animals is abnormal. Typically, 95% of the human cells in peripheral blood are B lymphocytes. To determine whether lentiviral vectors efficiently transduce stem cells that maintain normal hematopoiesis in vivo, we isolated Sca-1⁺c-Kit⁺Lin⁻ bone marrow cells from mice without 5-fluorouracil treatment, and transduced these cells in the absence of cytokine stimulation with a novel lentiviral vector containing a GFP (green flourescent protein) reporter gene. These cells were transplanted into lethally irradiated C57Bl/6 mice. In fully reconstituted animals, GFP expression was observed in 8.0% of peripheral blood mononuclear cells for 20 weeks posttransplantation. Lineage analysis demonstrated that a similar percentage (approximately 8.0%) of GFP-positive cells was detected in peripheral blood B cells, T cells, granulocytes and monocytes, bone marrow erythroid precursor cells, splenic B cells, and thymic T cells. In secondary transplant recipients, up to 20% of some lineages expressed GFP. Our results suggest that quiescent, hematopoietic stem cells are efficiently transduced by lentiviral vectors without impairing self-renewal and normal lineage specification in vivo. Efficient gene delivery into murine stem cells with lentiviral vectors will allow direct tests of genetic therapies in mouse models of hematopoietic diseases such as sickle cell anemia and thalassemia, in which corrected cells may have a selective survival advantage. *Stem Cells 2000;18:352-359*

INTRODUCTION

Efficient transduction of hematopoietic stem cells is essential for a genetic treatment of many blood diseases. Noncycling, quiescent [1] stem cells are poorly transduced with retroviral vectors because breakdown of the nuclear membrane during mitosis is required for efficient retroviral integration into host chromatin [2]. Poor transduction of stem cells is also correlated with low-level expression of receptors for amphotropic retroviral vectors [3]. Although adeno-associated viral (AAV) vectors have been shown to efficiently transduce postmitotic skeletal muscle or liver cells [4-6], transduction of hematopoietic stem cells is low [7]. In addition, we have demonstrated that virally transduced genes are silenced after integration into host chromosomes [8], and that the mechanism of silencing involves histone deacetylation and chromatin condensation [9].

In contrast to retroviruses, lentiviral vectors efficiently transduce quiescent postmitotic cells [10, 11]. In a recent study, *Miyoshi et al.* [12] demonstrated that human CD34⁺ cells are efficiently transduced by a lentiviral vector, and

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sustained expression of a green flourescent protein (GFP) reporter gene is detected in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice for 22 weeks. However, hematopoiesis in these animals is abnormal. The human cells in peripheral blood of these mice were predominantly B lymphocytes [12], and thus it is difficult to evaluate the maintenance of normal hematopoiesis after lentiviral transduction. We now report the efficient transduction of highly purified murine bone marrow stem cells with a novel lentiviral vector. We demonstrate that normal hematopoiesis is preserved after lentiviral transduction of stem cells and reconstitution of lethally irradiated recipient mice. In addition, we demonstrate that expression of transduced genes persists after long-term engraftment and in secondary transplants.

Materials and Methods

Purification of Stem Cells

C57Bl/6 Hbb^d donor mice were obtained from the Jackson Lab (Bar Harbor, ME; http://www.jax.org) and bred in our mouse facility. Bone marrow was flushed from femurs and tibia of 8- to 16-week-old donor mice with Iscove's modified Dulbecco's medium (IMDM) containing 5 mM EDTA, 2% fetal bovine serum (FBS) and antibiotics. After washing once with separation buffer (phosphate-buffered saline [PBS] containing 5 mM EDTA and 0.5% charcoal-treated bovine serum albumin), cells were labeled with biotin-conjugated Sca-1 antibody (Pharmingen; San Diego, CA; http://www.pharmingen.com) in label buffer (PBS containing 5 mM EDTA) for 15 min on ice. Cells were then washed once with separation buffer to remove free Sca-1 antibody, and labeled with magnetic bead-conjugated streptavidin (Miltenyi Biotech GmbH; Bergisch Gladbach, Germany; http://www.miltenyibiotec.com) for 15 min on ice. This step was immediately followed (without washing) by addition of fluorescein isothiocyanate-conjugated streptavidin (Caltag; South San Francisco, CA; http://www.caltag.com) for another 15 min on ice. After labeling, cells were washed once with separation buffer, and magnetic bead-labeled cells were enriched, using the maximum allowable concentration, on a column (Miltenyi) as the manufacturer suggested. Cells were eluted from the column, pelleted, and resuspended in labeling buffer. Cells were then simultaneously labeled with allophycocyanin-conjugated c-Kit antibody (Pharmingen) and a cocktail of phycoerythrin (PE)-conjugated lineage antibodies containing B-220, CD3, CD4, CD5, CD8, Mac-1, Gr-1, and Ter-119 (Pharmingen) for 15 min on ice. Cells were washed once with separation buffer and resuspended in IMDM for sorting on a Becton-Dickinson (Franklin Lakes, New Jersey; http://www.bd.com) FCASVantage SE. Sca-1^+c-Kit^+Lin^- cells were collected into a 5-ml tube with IMDM containing 1% FBS for transduction.

Production of Lentiviral Vector

To construct the pPCW-eGFP gene transfer vector, a polymerase chain reaction (PCR)-amplified DNA fragment containing the EGFP gene (derived from pEGFP-C1, Clontech Laboratories; Palo Alto, CA) was ligated into the BamHI/XhoI sites of the pHR-CMV-*lacZ* plasmid [10], generating pHR-cytomegalovirus (CMV)-eGFP. Then, a 150-bp sequence of DNA (coordinates 4327 to 4483) containing the central polypurine tract (PPT) and central terminal site (CTS) was PCR-amplified from the HIV-1 pSG3 molecular clone [13] and ligated into the unique ClaI site of pHR-CMV-eGFP. To increase eGFP expression in the transduced cells, a post transcriptional regulatory element derived from the woodchuck hepatitis virus (WPRE) was inserted downstream of eGFP, generating the pPCW-eGFP gene transfer vector.

Transduction of Stem Cells

Sorted Sca-1^+c-Kit^+Lin^- stem cells were centrifuged at $300 \times g$ for 10 min, and resuspended in IMDM containing 10 μg/ml dextran sulfate and 1% FBS. One thousand stem cells were infected in a total volume of 100 μl for 4 h at 37°C and transplanted into a single, lethally irradiated mouse as described below.

Transplantion

The congenic recipient mice (C57Bl/6 Hbb^s) were purchased from the Jackson Lab and maintained in our transgenic facility. Mice were lethally irradiated with 1,250 RADS in two doses of 625 RADS, each with a Picker Cyclops Cobalt-60 unit. Anesthetized mice were transplanted with 1,000 stem cells per mouse in 100 μl IMDM by retro-orbital injection. Transplants were maintained on antibiotic water containing 1.1 g/l neomycin sulfate (Sigma; St. Louis, MO; http://www.sigma-aldrich.com) and 1×10^6 units/l polymyxin B sulfate (Sigma) for two months posttransplantation. For secondary transplantation, five million unfractionated bone marrow cells from primary transplants were retro-orbitally injected into each recipient mouse. Hematopoietic recovery of transplants was monitored by analysis of diffuse hemoglobin using high performance liquid chromatography (HPLC) as described previously [14].

Mononuclear Cell GFP Analysis

Fifty microliters of peripheral blood from each mouse were collected from the tail vein and mixed with 1 ml PBS containing 2.5 mM EDTA. The cells were further diluted to 3 ml with PBS immediately before gradient separation. Three milliliters of Histopaque-1077 (Sigma) were loaded into a 15-ml conical tube, and 3 ml of diluted blood cells were carefully layered on the top. Cells were centrifuged at $300 \times g$ for 10 min. The opaque layer of mononuclear cells that formed at the interface was carefully transferred to a new tube and washed

once with PBS. Cells were then aliquoted and labeled with PE-conjugated antibodies as described above, and GFP expression was analyzed using fluorescence-activated cell sorting (FACS). The same gradient procedure was also used to prepare bone marrow mononuclear cells for analysis. Single cell suspensions of spleen or thymus were used directly for labeling and analysis.

Progenitor Assay

Bone marrow cells were mixed with methylcellulose medium M3434 (Stem Cell Technology; Vancouver, BC; http://www.stemcell.com) to 3×10^4/ml, plated onto 35-mm plates, and cultured at 37°C for 12 days as the manufacturer suggested. Colony-forming units-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) colonies were examined using an inverted microscope, and fluorescent images from the colonies were captured using an Olympus IX70 inverted microscope with epifluorescence optics and a Hamamatsu charged-coupled device camera.

Results

Reconstitution of Lethally Irradiated Mice with Sca-1$^+$c-Kit$^+$Lin$^-$ Cells

Bone marrow was isolated from femurs and tibias of C57BL/6 donor mice containing the diffuse hemoglobin (Hbb^d) haplotype. No 5-fluorouracil was administered to the mice to mobilize stem cells prior to marrow isolation. Sca-1$^+$c-Kit$^+$Lin$^-$ cells were isolated as described in the **Materials and Methods** section and transplanted into lethally irradiated, wild-type C57Bl/6 recipient mice containing the single hemoglobin (Hbb^s) haplotype. As few as 50 of these highly purified cells were capable of fully reconstituting hematopoiesis (data not shown). Reconstitution with donor stem cells was followed by HPLC of hemolysates. For the transduction experiments described below, 1,000 Sca-1$^+$c-Kit$^+$Lin$^-$ cells per recipient mouse were routinely infected with a lentiviral vector prior to transplantation. Figure 1 illustrates reconstitution in a representative animal transplanted with 1,000 transduced stem cells. Within eight weeks posttransplantation, all erythroid cells were derived from the donor as indicated by the replacement of Hbb^s (β^s, β^t) with Hbb^d (β^{maj}, β^{min}).

Design of the Lentiviral Vector

The lentiviral vector used in this study was an HIV-1 vector pseudotyped with vesicular stomatitis virus G (VSV-G) glycoprotein. The vector contained a CMV promoter driving a GFP reporter gene, a central PPT and CTS derived from a molecular clone of HIV-1 to increase packaging efficiency [13], and a posttranscriptional regulatory element derived from WPRE (Fig. 2A). *Zufferey et al.* [15] recently demonstrated that the WPRE enhances retroviral and lentiviral transduction efficiency in cultured cells by increasing the efficiency of RNA processing. In our experiments, lentiviral vectors with or without WPRE were able to transduce cultured murine erythroleukemia cells efficiently (data not shown); however, only the vector with a WPRE efficiently transduced purified murine bone marrow stem cells as described below. We were unable to detect any GFP expression in hematopoietic cells in vivo when using the vector without WPRE (data not shown).

Transduction of Sca-1$^+$c-Kit$^+$Lin$^-$ Cells and Persistent Expression of Vector-Derived GFP in Hematopoietic Lineages in Vivo

Although lentiviral vectors are able to transduce quiescent cells [10, 11], transduction efficiency is enhanced when

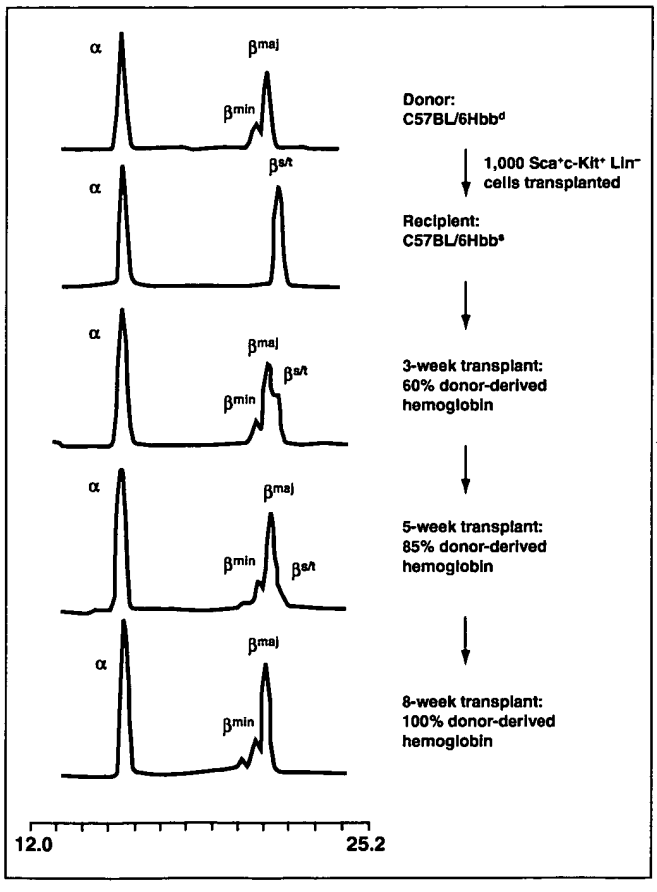

Figure 1. Reconstitutiton of lethally irradiated mice with Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells. *Lethally irradiated recipient mice were transplanted with 1,000 virally transduced Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells. Peripheral blood was collected from donor (diffuse hemoglobin haplotype), recipient (single hemoglobin haplotype), and transplants at indicated time points. Hemolysates were analyzed by HPLC using a 35% to 41% acetylnitrile gradient. Full reconstitution was achieved eight weeks after transplantation.*

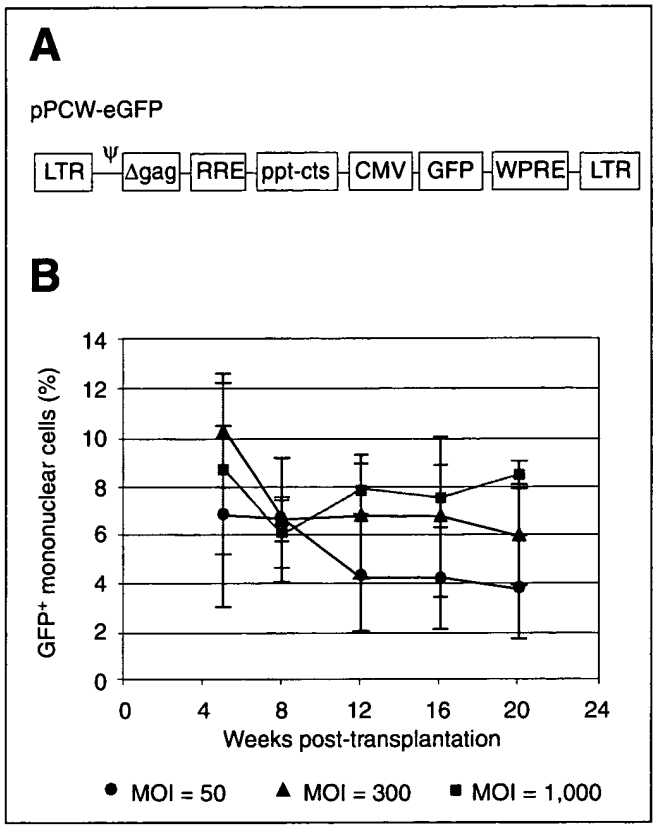

Figure 2. Stable transduction of Sca-1⁺c-Kit⁺Lin⁻ stem cells by a lentiviral vector. *(A) Map of lentiviral vector PCW-eGFP used in this study. A central PPT and a CTS derived from a molecular clone of HIV-1 were inserted in the vector to increase packaging efficiency. A post-transcriptional regulatory element of WPRE was placed downstream in the sense orientation with CMV/eGFP to increase GFP expression. (B) Persistence of GFP expression in mononuclear cells from peripheral blood. One thousand Sca-1⁺c-Kit⁺Lin⁻ stem cells were transduced with the vector at an MOI of 50, 300, or 1,000, and these cells were transplanted into a single, lethally irradiated mouse. Four mice were reconstituted with stem cells infected at an MOI of 300 and four at an MOI of 1,000. Three mice were reconstitutied with cells infected at an MOI of 50. At the time points indicated, 50 ul of blood from each transplanted mouse were collected from the tail vein. Mononuclear cells were then isolated and analyzed for GFP expression by FACS. Starting at 16 weeks posttransplantation, some mice were sacrificed for secondary transplantation and bone marrow cell analysis. At least two mice from each group (MOI= 50, 300, or 1,000) were analyzed after 20 weeks. GFP expression persisted for 20 weeks in all reconstituted mice. These data demonstrate that lentiviral vectors mediate stable transduction of stem cells which are capable of long-term reconstitution in vivo. The results also demonstrate that high MOIs are required for efficient lentiviral transduction of hematopoietic stem cells.*

cells are induced to enter the cell cycle [16, 17]. However, induction of hematopoietic stem cell replication in vitro may result in loss of pluripotency. Therefore, we chose to infect purified stem cells for only 4 h in the absence of cytokines. Cells were then transplanted into lethally irradiated recipients in which stem cells could home to the marrow and replicate in vivo under conditions that favor maintenance of pluripotency. Transduction efficiency was measured by FACS analysis for GFP expression.

Figure 2B illustrates the results of GFP expression in peripheral blood mononuclear cells of mice at 5 to 20 weeks posttransplantation. These animals received stem cells transduced at multiplicities of infection (MOIs) of 50, 300 or 1,000. Full reconstitution with donor stem cells was achieved at eight weeks (data not shown). At an MOI of 50, an average of 4% of peripheral blood mononuclear cells were GFP-positive, and the percentage was increased to 6% and 8% with MOIs of 300 and 1,000, respectively. Few GFP-positive mononuclear cells were detected when stem cells were transduced with an MOI of 5 (data not shown). These results demonstrate that high MOIs are required for efficient lentiviral transduction of hematopoietic stem cells. The data also demonstrate that this lentiviral vector mediates stable transduction of stem cells that are capable of long-term reconstitution in vivo.

We next examined whether transduced hematopoietic stem cells maintained pluripotency after long-term reconstitution. Mice were sacrificed at 16 and 20 weeks posttransplantation and mononuclear cells were labeled with PE-conjugated, lineage-specific antibodies for B cells (α-B220), T cells (α-CD3, CD4, and CD8), neutrophils, monocytes and granulocytes (α-Mac-1 and GR-1), and erythroid cells (α-Ter-119). Results from a representative mouse are shown in Figure 3. In peripheral blood, 7.9% of B cells, 9.6% of T cells, and 12.6% of neutrophils, granulocytes, and monocytes were positive for GFP expression (Fig. 3A). Similar values were observed at all time points for peripheral blood mononuclear cells (data not shown). These results demonstrate that transduced hematopoietic stem cells maintain the capacity for normal lineage specification in fully reconstituted mice.

Interestingly, less than 1% of erythroid cells in peripheral blood were positive for GFP (data not shown). Although this result was surprising, we speculated that erythroid progenitors were stably transduced, but that little GFP persists in enucleated red blood cells. Therefore, we examined Ter-119⁺ bone marrow mononuclear cells for GFP expression and observed that 9.7% of these erythroid precursors were GFP-positive. This result demonstrated that transduced stem cells are also capable of normal erythroid lineage differentiation. Bone marrow B cells, neutrophils, granulocytes, and monocytes (Fig. 3B), as well as splenic B cells and thymic T cells (Fig. 3C) were all GFP-positive at similar percentages. Again, these results demonstrate that lentiviral vectors efficiently transduce hematopoietic stem cells and do not alter normal properties of self-renewal and lineage specification in fully reconstituted mice.

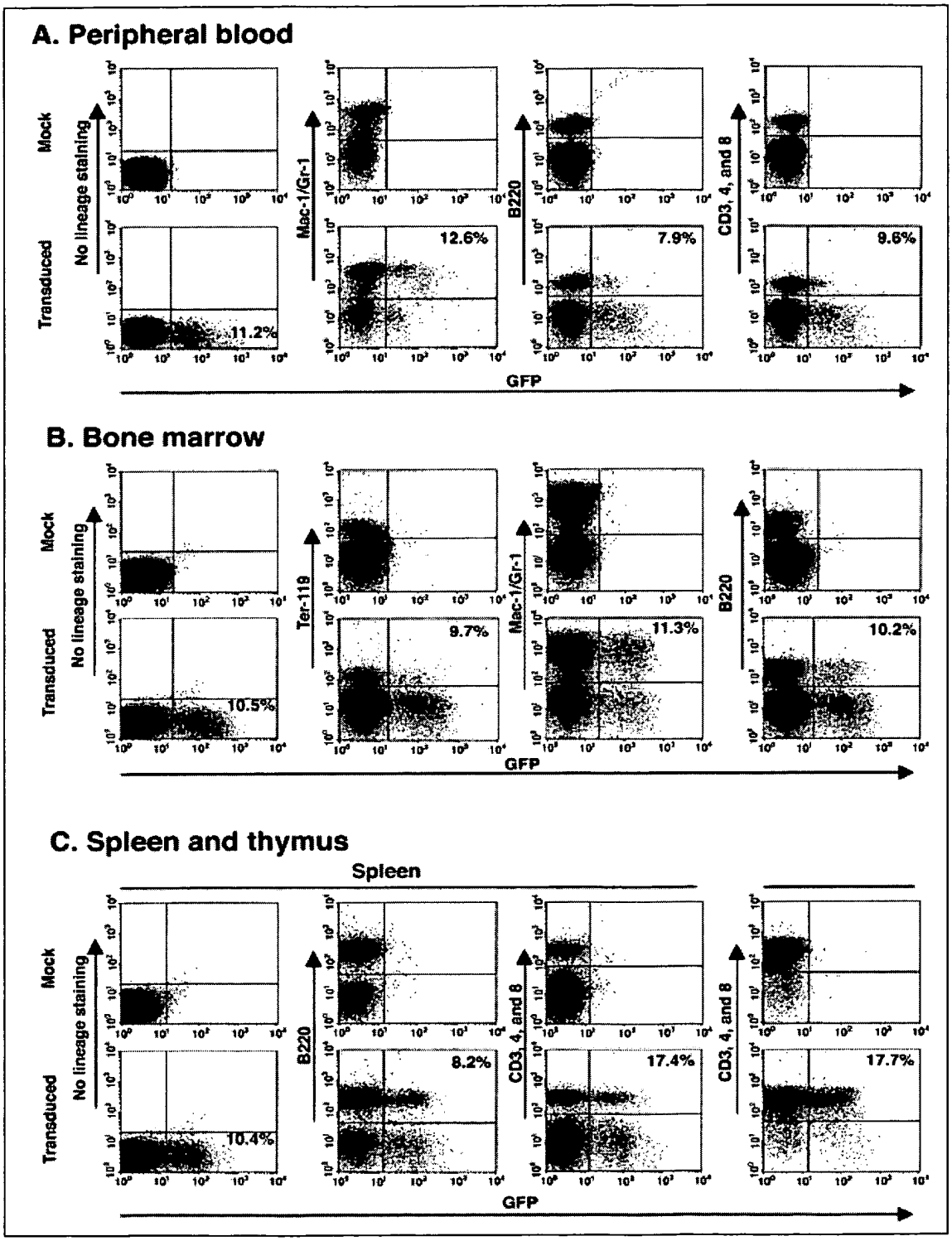

Figure 3. GFP expression in hematopoietic cell lineages. At 16 weeks posttransplantation, mononuclear cells were collected from peripheral blood (A), bone marrow (B), and spleen and thymus (C). This primary recipient initially received 1,000 stem cells transduced at an MOI of 300. Mononuclear cells were analyzed by FACS without staining or after staining with PE-conjugated lineage antibodies (B220 for B cells, a mixture of CD 3, 4, and 8 for T cells, a mixture of Mac-1 and Gr-1 for neutrophils, monocytes, and granulocytes, and Ter-119 for erythroid cells). The percentage of GFP-positive mononuclear cells is shown on each FACS profile. These results demonstrate that transduced hematopoietic stem cells maintain the capacity for normal lineage specification in fully reconstituted mice.

Table 1. GFP expression in peripheral blood mononuclear cells of secondary transplants

	Secondary transplant							Primary transplant
	TP85	TP86	TP87	TP88	TP89	TP90	Average	TP64
No staining	10.8	12.4	14.3	8.4	4.1	9.8	10.0	11.2
B220	7.2	5.8	9.7	3.9	3.0	7.8	6.2	7.9
CD3, 4, and 8	16.3	19.3	20.8	12.4	7.1	15.4	15.2	9.6
Mac-1/Gr-1	6.1	12.6	7.6	8.0	3.5	5.9	7.3	12.6

Five million bone marrow cells from a 16-week primary transplant were used to reconstitute lethally irradiated secondary recipients. The percentages of GFP-positive cells in peripheral blood mononuclear cells, unstained or stained with lineage-specific antibodies, are shown for six secondary transplants at 12 weeks posttransplantation. The percentage of GFP-positive, peripheral blood mononuclear cells in the primary recipient at 16 weeks posttransplantation is also shown for comparison.

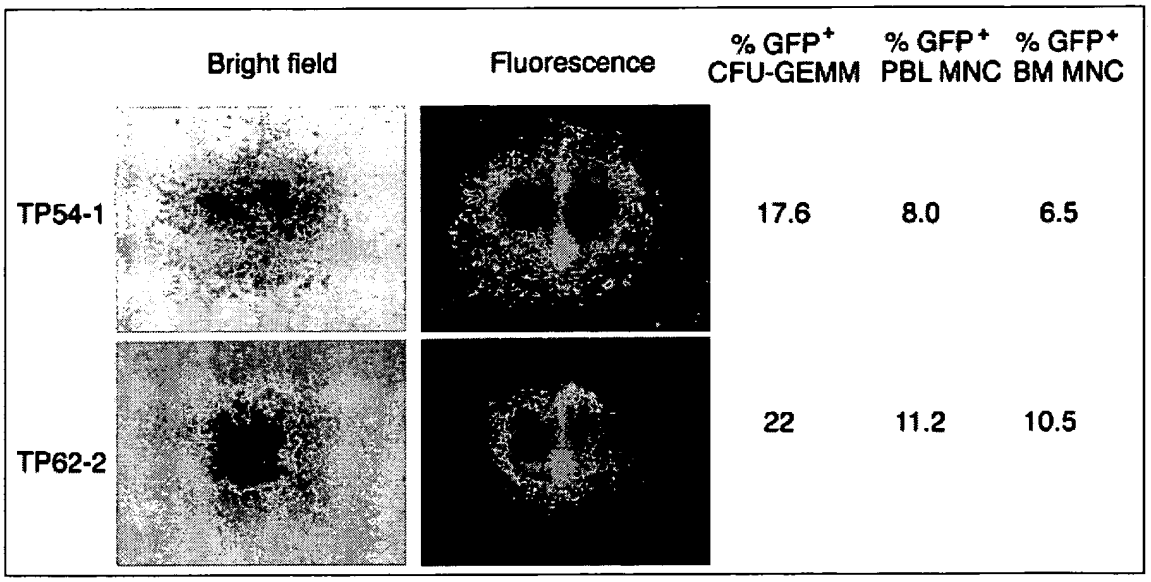

Figure 4. GFP expression in CFU-GEMM colonies. *Bone marrow cells (3×10^5 per 35-mm plate) from fully reconstituted recipient mice at 16 weeks (mouse #TP 54) or 20 weeks (mouse #TP 62) posttransplantation were plated in methylcellulose and cultured for 12 days at 37°C. CFU-GEMM colonies were scored and examined by fluorescence microscopy. TP 54 and TP 62 each received 1,000 stem cells that were transduced at an MOI of 1,000 and 300, respectively. The percentages of GFP-positive CFU-GEMM colonies from the two recipients are shown. GFP-positive mononuclear cells from peripheral blood and bone marrow of the same mice are also shown for comparison. Colonies were either fully positive or negative; no sectoring was observed. This result suggests that silencing of the transduced gene does not occur during lineage specification. However, some silencing may occur in early progenitors. The percentage of GFP-positive CFU-GEMM was approximately two times higher than the percentage of GFP-positive mononuclear cells in peripheral blood and bone marrow. Nevertheless, a high percentage of GFP-positive cells persists for 16 and 20 weeks in fully reconstituted mice*

Persistent GFP Expression in Secondary Transplants

To determine whether GFP expression persists in secondary transplants, five million bone marrow cells from primary transplants were injected into lethally irradiated C57BL/6Hbb^d recipient mice. Table 1 illustrates one set of secondary transplants derived from a 16-week primary transplant. GFP expression was detected in all reconstituted secondary recipient mice at 12 weeks posttransplantation, and the average percentages of GFP-positive mononuclear cells, B cells, T cells, and neutrophils and monocytes were consistent with those from the primary transplant. These results further support the conclusion that long-term, self-renewing stem cells were transduced by the lentiviral vector and that the pluripotency of these cells was preserved in the fully reconstituted recipients.

Silencing of Lentivirally Transduced Genes

We also examined GFP expression in CFU-GEMM derived from long-term reconstituted mice (Fig. 4). Bone marrow cells were obtained from animals at 16 weeks posttransplantation and

plated (3×10^4/plate) in methylcellulose. Colonies were counted after 12 days of culture. As expected, the number of CFU-GEMM derived from reconstituted and wild-type mice was similar (data not shown). Interestingly, the GFP-positive colonies were not sectored into expressing and non-expressing cells (Fig. 4); the colonies were either completely positive or negative. In transgenic mice that express the *lacZ* gene specifically in erythroid cells, early erythroid colonies are sectored into LacZ expressing and nonexpressing cells [18]. This result in transgenic mice suggested that silencing of the transgene occurred during erythroid differentiation. The colonies derived from mice that were transduced with the lentiviral vector were not sectored; therefore, silencing of the transduced gene apparently does not occur during lineage specification. However, silencing may occur in stem cells or early progenitors. PCR analysis demonstrated that 40% of CFU-GEMM contained GFP DNA (data not shown); however, only 20% of these colonies and 10% of peripheral blood cells expressed GFP. These data suggest that silencing occurs at two separate stages after transduction. One-half of all vector integration sites are silenced in stem cells soon after transduction. Subsequently, one-half of the remaining sites are silenced in early progenitors. Nevertheless, a high percentage (10%) of GFP-positive cells persist in bone marrow and peripheral blood for 20 weeks posttransplantation in fully reconstituted mice and for at least 12 weeks in secondary recipients.

Discussion

We have demonstrated that highly purified murine bone marrow stem cells are efficiently transduced with a novel lentiviral vector and that the normal pluripotency of these cells is preserved in fully reconstituted, lethally irradiated mice. Although efficient transduction of human CD34$^+$ cells has been reported previously [12], this is the first report to our knowledge of stable transduction of murine hematopoietic stem cells with a lentiviral vector. In the system reported here, normal hematopoiesis is maintained after transplantation; therefore, we can conclude that stable lentiviral transduction does not alter normal cell lineage specification. The CD34$^+$ cells transduced previously were transplanted into NOD/SCID mice in which normal hematopoiesis is not maintained; therefore, it is difficult to determine whether normal lineage specification is altered by lentiviral transduction.

Interestingly, high MOIs were required for efficient transduction in our experiments. At an MOI of 5, few cells were transduced. An MOI of 50 was required for significant transduction and an MOI of 1,000 increased the efficiency of transduction twofold. High MOIs were not required to transduce human CD34$^+$ cells in the experiments cited above [12]. The high MOIs required in the present studies may result from the use of highly purified stem cells that were transduced without cytokine stimulation. We used unstimulated stem cells in an attempt to preserve pluropotentiality, and these undifferentiated cells may express fewer receptors for the VSV-G glycoprotein.

Persistent expression of virally transduced genes has been problematic for many viral vectors. Our previous studies have demonstrated that histone deacetylation and chromatin condensation are involved in the silencing of virally transduced genes [8, 9]. In several cases expression of transduced genes was completely extingished. In the present studies stable expression was established in 10% of hematopoietic stem cells. Apparently, lentiviral vectors are not as sensitive to silencing as AAV and Moloney-based retroviral vectors. However, some silencing does occur. Analysis of bone marrow obtained from long-term reconstituted mice demonstrates that 40% of GEMM colonies contain the transduced gene, but the gene is expressed in only 10% of peripheral blood mononuclear cells. Similar results were observed when human CD34$^+$ cells were transduced with a lentiviral vector and transplanted into NOD/SCID mice [12]. We are presently determining whether genes that are transduced by lentiviral vectors can be reactivated by histone deacetylase inhibitors in vivo.

Efficient gene delivery into murine hematopoietic stem cells will provide a powerful tool for genetic correction of thalassemia and sickle cell disease in mouse models [14, 19-21] and will provide a foundation for similar protocols in man. The life span of sickle and thalassemic red blood cells is significantly shorter than normal; therefore, we speculate that correction of approximately 10% of erythroid precursors in the marrow may translate into a major fraction in the peripheral blood. If silenced, lentivirally transduced genes can be reactivated by deacetylase inhibitors, a combination of lentiviral transduction and drug treatment may result in high-level, therapeutic gene expression and thus provide a powerful treatment for hereditary blood diseases.

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Dr. T.T. holds equity in Erythrogen, Inc. and *Drs. J.K.* and *Z.W.* hold equity in Transzyme, Inc.

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- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
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- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
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